The remarkable vocal anatomy of the koala (Phascolarctos cinereus): insights into low-frequency sound production in a marsupial species

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The remarkable vocal anatomy of the koala (*Phascolarctos cinereus*): insights into low-frequency sound production in a marsupial species

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Abstract

Koalas are characterised by a highly unusual vocal anatomy, with a descended larynx and velar vocal folds, allowing them to produce calls with disproportionately low frequencies. Here we use advanced imaging techniques, histological data, classical macroscopic dissection and behavioural observations to provide the first detailed description and interpretation of male and female koala vocal anatomy. We show that both males and females have an elongated pharynx and soft palate, resulting in a permanently descended larynx. In addition, the hyoid apparatus has a human-like configuration in which paired dorsal, resilient ligaments suspend the hyoid apparatus from the skull, while the ventral parts tightly connect to the descended larynx. We also show that koalas can retract the larynx down into the thoracic inlet, facilitated by a dramatic evolutionary transformation of the ventral neck muscles. First, the usual retractors of the larynx and the hyoid have their origins deep in the thorax. Second, three hyoid muscles have lost their connection to the hyoid skeleton. Third, the genioglossus and geniohyoid muscles have greatly increased in length. Finally, the digastric, omohyoid and sternohyoid muscles, connected by a common tendinous intersection, form a guiding channel for the dynamic down-and-up movements of the ventral hyoid parts and the larynx. We suggest that these features evolved to accommodate the low resting position of the larynx and assist in its retraction during call production. We also confirm that the edges of the intra-pharyngeal ostium have specialised to form the novel, extra-laryngeal velar vocal folds, which are much larger than the true, intra-laryngeal vocal folds in both sexes, but more developed and specialised for low frequency sound production in males than in females. Our findings illustrate that strong selection pressures on acoustic signalling not only lead to the

specialisation of existing vocal organs, but can also result in the evolution of novel vocal structures in both sexes.

Introduction

Examining vocal anatomy is an important step towards understanding the proximate factors that shape the vocal signals of a given species. The link between vocal anatomy and vocal output has been thoroughly investigated in humans (e.g. speech production and operatic singing; Némai & Kelemen, 1933; Doscher, 1994; Titze, 1994; Lieberman et al, 2001; Dayme, 2009; Fitch, 2010; Koda et al, 2012). However, over the last 25 years the generalisation of the source-filter theory has also allowed researchers to explicitly link vocal anatomy and acoustic output in non-human mammals. For example, studies combining behavioural, morphological and acoustic data have revealed that red deer (*Cervus elaphus*) and fallow deer (*Dama dama*) have a descended and retractable larynx that lowers vocal tract resonances in their respective sexual calls (Fitch & Reby, 2001, McElligott et al 2006), that male saigas (*Saiga tatarica*) and southern elephant seals (*Mirounga leonina*) extend the nasal tract to lower resonance frequencies in their rutting calls (Frey et al, 2007, Sanvito et al 2007) and have also provided key insights into sound production in Savanna elephants (*Loxodonta africana* - Herbst et al 2012, 2013), North American wapitis (*Cervus canadensis* - Reby et al, 2016), and koalas (*Phascolarctos cinereus* - Charlton et al 2011, 2013).

According to the source-filter theory, mammals produce vocal signals via a two-stage process (Fant, 1960): firstly, a source signal is generated as air expelled from the lungs passes through the larynx, causing the vocal folds to open and close, and breaking up the airstream to produce a sound wave. The rate that the vocal folds open and close determines the fundamental frequency (F0), responsible for the perceived

63 pitch of the vocalisation. The supra-laryngeal vocal tract then acts as a resonator,
64 selectively diminishing certain frequencies of the source signal and enhancing other
65 frequencies termed formants before the filtered signal radiates out through the mouth
66 or nostrils. Because source and filter characteristics of mammal vocal signals are
67 produced by the larynx and vocal tract, respectively; variation in these features can be
68 directly predicted from the laryngeal and vocal tract anatomy of the caller (for a review
69 see: Taylor et al 2016).

70 The koala (*Phascolarctos cinereus*) is a specialized arboreal folivore that inhabits
71 the open forests and woodlands of eastern and southeastern Australia dominated by
72 trees of the genus *Eucalyptus* (Lee and Carrick, 1989). Koalas are the only mammals
73 that consume eucalypt foliage as a dominant component of their diet (Tyndale-Biscoe,
74 2005). This relatively low-energy diet means that koalas spend a large proportion of
75 their time resting, only coming to the ground for brief periods in order to move between
76 trees (Martin and Handasyde, 1999). During the annual breeding season, however,
77 both sexes roam over much larger areas in order to locate a suitable mating partner
78 and vocal activity increases (Ellis et al, 2009). In particular, males produce a high rate
79 of extremely low-pitched bellow vocalizations when most copulations are predicted to
80 occur (Ellis et al, 2011), indicating that these calls are important in reproductive
81 contexts. Bellows are produced in a characteristic two-stage calling posture, gradually
82 changing from pronounced extension to marked flexion of the head and neck region
83 (Charlton et al, 2011; Fig. 1).

84 Recent studies examining the information content and function of male koala
85 bellows have shown that formants are important cues to male identity and size that are
86 used in both inter- and intra-sexual contexts (Charlton et al, 2011a; Charlton et al,
87 2011b; Charlton et al, 2012c; Charlton et al, 2012a; Charlton et al, 2012b; Charlton et

al, 2013). Taken together, these findings indicate that identity- and size-related formant information is functionally relevant to male koalas in their natural environment, and that sexual selection favours males able to produce lower formants in their bellows. Interestingly, the formant pattern of male bellow exhalation phases and this species' well-developed sternothyroid muscle indicate that koalas extend the vocal tract just prior to call production by lowering the larynx (Charlton et al, 2011b). In addition, anatomical studies have revealed that male koalas have an additional set of vocal folds outside of the larynx, the 'velar vocal folds' (VVFs), which are used instead of the intra-laryngeal vocal folds to produce the extremely low F0 of bellow vocalisations (Charlton et al, 2013). As the VVFs are much larger than the laryngeal vocal folds they can oscillate periodically at much lower frequencies (Charlton et al, 2013).

Female koalas produce bellow vocalizations when they are in oestrous (Smith, 1980) and it has been shown that they also possess adaptations of the soft palate (velum) similar to those found in males (Curry et al 2016). Female koala bellows have a mean F0 of 31.3 Hz (Charlton, 2015) which suggests that female koalas, like males, use VVFs to produce disproportionately low-pitched calls, although not as low as those of males. The formant spacing of the inhalation sections of female bellows is 423.5 Hz (Charlton, 2015). Applying the following equation $eVTL = c/2\Delta F$, in which eVTL is the estimated vocal tract length of the caller, c = the speed of sound in warm air (350 m/s) and ΔF is the formant spacing, yields an estimated vocal tract length of 41.3 cm. This is clearly a much longer vocal tract than expected for an animal the size of a koala, and indicates that female koalas, like males, possess adaptations that allow them to elongate their vocal tract. Female koalas also produce individually distinctive squawks, screams, squeaks and wails (collectively termed "rejection calls") when they rebuff male copulation attempts (Charlton, 2015). The mean F0 of these calls (792.1 Hz) is

substantially higher than the mean F0 of the inhalation sections of female bellows (31,3 Hz – Charlton 2015), indicating that the rejection calls are more likely to be produced by the laryngeal vocal folds.

In this study, we combined histological investigations with measurement data derived from dissections and computed tomography (CT) imaging to comprehensively document male and female koala vocal anatomy. Our specific aims were to: 1) provide a detailed description of the koala's vocal anatomy; 2) identify key differences in laryngeal and vocal tract anatomy between the sexes; 3) examine the tissue composition of the koala's remarkable velar vocal folds; and 4) determine *in situ* whether male koalas can retract their larynx into the thoracic inlet. Our findings will provide additional insights into the production mechanisms of male and female koala vocal signals.

Material and Methods

Specimens

Whole-body specimens: One adult female and one adult male koala (weighing 5.9 and 7.6 kg, and having overall body lengths of 680 and 750 mm, respectively) were dissected. The animals were euthanized as part of normal hospital practice at Moggill Koala Hospital, Bellbowrie, Queensland, Australia. A second adult male was used for demonstrating the 'retracted' larynx in a virtual 3D reconstruction based on a CT scan (S-Tab. 1). The specimens were stored deep-frozen (-20° C) before shipment to the Leibniz Institute for Zoo and Wildlife Research (IZW), Berlin, Germany, for anatomical investigations. No animals were killed expressly for the purpose of this study.

136 Excised larynges: A total of 18 excised larynges (9 male, 9 female), including the
137 part of the soft palate with the intra-pharyngeal ostium, were collected from dead and
138 euthanized individuals at Moggill Koala Hospital during the 2011 breeding season
139 (October-December). The specimens were stored deep-frozen (-20° C) before
140 shipment to the Leibniz Institute for Zoo and Wildlife Research (IZW), Berlin, Germany,
141 for anatomical investigations. In addition, the larynges of the two dissected whole body
142 specimens (1 male, 1 female) were included in this series. For measurements, the
143 larynges were individually defrosted and mediosagittally cut into two halves. Larynx
144 and vocal fold dimensions were measured in the half-larynx specimens whereas velar
145 vocal fold dimensions were measured in the undivided soft palate parts. Overall
146 dimensions of the larynx and velar vocal folds were documented photographically.
147

148 ***Computer tomographic (CT) and anatomical investigations***

149 To obtain as much information as possible about skeletal and soft tissue prior to
150 our macroscopic dissections, the male and female deep-frozen specimens were
151 scanned using a 64-slice spiral Computer Tomograph Aquilion CX (Toshiba Medical
152 Systems Corp., 1385 Shimoishigami, Otawara-shi, Tochigi 324-8550, Japan) at the
153 IZW. The post-mortem *in situ* positioning of the vocal anatomy was documented using
154 virtual serial sections (MPRs obtained with the settings: 120.0 kV, 120.0 mA, slice
155 thickness 0.6mm) and 3D-reconstructions established by Vitrea 2 software. A second
156 male was slowly defrosted in a refrigerator at +5° C over a period of 48 hours. Once
157 the specimen had fully thawed, its larynx was pushed back into the thoracic inlet by
158 external manipulation and fixed using a plastic strap. It was then refrozen and the
159 plastic strap was removed in order to CT scan with the larynx in a fully retracted state
160 (Fig. 2).

For all anatomical dissections conducted at the IZW the specimens were thawed at +5°C for a period of 24-48 hours and then submersed in water to facilitate macroscopic dissection and photographic documentation. This technique improves the diagnosis of structural details whilst also allowing for the dissection of non-preserved fresh specimens over a comparably long time period (cf. Frey et al 2007, 2008, 2011). The water in the dissection tank was continuously replaced by cool water (about 16°C) between consecutive dissection steps. Photographs were taken whilst the specimen was placed in a larger tank (using a Nikon D70s digital camera, Nikon Corp., Tokyo, Japan) and transferred to a computer for image processing (Adobe Photoshop 5.5 and CS4). Specimens were kept overnight in water at temperatures ranging between +2 and +4°C (refrigerator: Philipp Kirsch GmbH, Okenstrasse 103, 77652 Offenburg, Germany).

The specimens were dissected starting from the left side, and the anatomical structures were removed layer by layer. The right side was dissected in an identical manner. The larynges, head-and-neck glands, most muscles, hyoid apparatus, soft palate, tongue, ears and the trachea of the specimens were all fixed in 4% formalin and stored in the IZW morphological collection. The skeletal parts were cleaned and also stored in the IZW morphological collection. Photos of consecutive dissection steps were taken with a Nikon D70S digital camera (Nikon Corp., Tokyo, Japan) on a CF card, fed to a PC and processed with Adobe Photoshop 5.5 and CS4 (Adobe Systems Inc., San Jose, CA, USA).

Micro-CT investigation of excised larynges

In order to obtain *in-situ* information on laryngeal cartilages and soft tissues, one male and one female excised larynx (both earlier cut into two halves for

measurements) were subjected to Micro-CT investigation prior to invasive macroscopic dissection. The deep-frozen larynges were slowly defrosted at about 5° C for approximately 24h, and put into 4% formalin for 3 days to achieve soft fixation and avoid autolysis during the long scanning procedure. Subsequent storage in Phosphate Buffered Saline (PBS) for 5 days served to wash out the formalin. Immediately before scanning, a larynx was transferred to a 50 ml falcon tube filled with FomblinY®Perfluoropolyether lubricant (Solvay GmbH, Hannover, Germany. This oil is MR-inert, thus giving contrast only to the tissue, and avoids dehydration of the specimen during scanning. The scanning was conducted using a 7T PharmaScan 70/16 US (Bruker BioSpin, Ettlingen, Germany) with Bruker software Paravision 5.1 and a linear 38 mm volume resonator (Bruker BioSpin). Scanning was performed with the following settings: T2_TurboRARE_3D with TR/TE = 1500/32.5ms, rare factor 6, averages 6; FOV (Field of View) = 46 x 30 x 25.8mm; Matrix size = 306 x 200 x 172; image resolution = 150 µm x 150 µm x 150 µm; Scan time for the male and female larynx: 14h11m24s, respectively.

Histology

Following *in-situ* dissection in water, the VVFs of the whole body male specimen were excised and preserved in 10% neutral buffered formalin. Longitudinal and cross sections (5 µm in thickness) of the VVFs were then stained with haematoxylin-eosin for a general overview, Masson/Goldner Trichrome as a collagen fibre stain, and Elastica-Van Gieson as an elastic fibre stain. Photographs of representative histological slides were taken with a Keyence VHX 1000 Digital Microscope (Keyence Deutschland, Neu-Isenburg, Germany) using a VH-Z00R, RZ ∞ - 50, Zoom 5-50x lens

and a VH-Z20R, RZ x20-x200 lens. Photoshop version CS4 was used to process the images.

Laryngeal retraction

At key stages of the dissection, the larynx was manually retracted to document laryngeal mobility and extension of the pharynx, including the soft palate and the velar vocal folds. Retraction was achieved by fastening a string to the caudal end of the trachea, manually pulling the string through an opening in the abdominal wall, and attaching the string to the wall of the dissection tub using a clamp. Simultaneously, the head and neck were extended and pulled forward by a string fastened to an incisor tooth and attached to the opposite wall of the dissection tub by using another clamp.

Once the dissections were complete, the specimens were subjected to bacterial skeletonization. For this purpose, the skeletal parts were first put into appropriately sized tubs filled with cold water for several days to wash the blood out. The specimens were then transferred to a heated tank filled with water and kept at 38°C until the soft parts had been fully decomposed by bacteria. The bacterial skeletonization process took approximately one month. After this, the remaining bones were kept in the same tank at the same temperature in a concentrated solution of washing powder for an additional month before the skeletal parts were cleaned, dried, and used for graphic 2D-reconstructions.

Anatomical terms are in accordance with Nomina Anatomica Veterinaria (Schaller 2012; NAV, 2005, 2012).

Abbreviations

Adit. lar.	=	laryngeal entrance
App. hyo.	=	hyoid apparatus
Arc. pal. phar.	=	palatopharyngeal arc
Arc. zyg.	=	zygomatic arc
Basih.	=	basihyoid
Bifurc.	=	tracheal bifurcation
Bul. tymp.	=	tympanic bulla
Cart. aryt.	=	arytenoid cartilage
Cart. cost. IV	=	4th costal cartilage
Cart. cric.	=	cricoid cartilage
Cart. postaryt.	=	postarytenoid cartilage
Cart. proaryt.	=	proarytenoid cartilage
Cart. thyr.	=	thyroid cartilage
Cart. trach. I, IV	=	1st , 4th tracheal cartilage
Cav. nasi	=	nasal cavity
Cav. or.	=	oral cavity
Ceratoh.	=	ceratohyoid
Choan.	=	choanae (internal nares)
Clav.	=	clavicle
Cont. Mand.	=	contour of lower jaw
Cor	=	heart
Corn. caud.	=	caudal horn of thyroid cartilage
Corn. rostr.	=	rostral horn of thyroid cartilage
Cost. I , III, IV	=	1st , 3rd , 4th rib
Crist. med.	=	dorsomedian crest of soft palate
C1, C7	=	1st, 7th cervical vertebra
Diaphr.	=	diaphragm
Epigl.	=	epiglottis
Intsect. tend.	=	tendinous intersection

263	IPO	=	intra-pharyngeal ostium
264	Lar.	=	larynx
265	Lig. hyo:	=	hyoid ligament
266	Ling.	=	tongue
267	LVFs	=	laryngeal vocal folds
268	Man. sterni	=	sternal manubrium
269	Meat. ac. ext.	=	external acoustic meatus
270	M. ceratoh.	=	ceratohyoid muscle
271	M.cricphar.	=	cricopharyngeus muscle
272	M. digastr.	=	digastric muscle
273	M. digastr. vent. caud.	=	caudal belly of digastric muscle
274	M. digastr. vent. rostr.	=	rostral belly of digastric muscle
275	M. genioh.	=	geniohyoid muscle
276	M.geniogllar.	=	genioglossolaryngeal muscle
277	M. hyoceph.	=	hyocephalic muscle (united M. styloh.,
278			M. stylphar.caud., M.hyophar.)
279	M. hyogl. (sin.)	=	(left) hyoglossus muscle
280	M. hyophar.	=	hyopharyngeus muscle
281	M. omoh.	=	omohyoid muscle
282	M. parotaur.	=	parotidoauricularis muscle
283	M. sternceph.	=	sternocephalic muscle
284	M. sternoh. (sin., dex.)	=	(left, right) sternohyoid muscle
285	M. sternthyr. (sin., dex.)	=	(left, right) sternothyroid muscle
286	M. stylogl.	=	styloglossus muscle
287	M. styloh.	=	stylohyoid muscle
288	M. stylphar. caud.	=	caudal stylopharyngeal muscle
289	M. thyroh.	=	thyrohyoid muscle
290	M. thyrphar	=	thyropharyngeus muscle
291	M^4	=	4^{th} upper molar
292	Nar. (sin.)	=	(left) nostril
293	Nasal vt.	=	nasal vocal tract

Nasophar.	=	nasopharynx
Oesoph.	=	oesophagus
Oral vt.	=	oral vocal tract
Orophar.	=	oropharynx
Os pteryg.	=	pterygoid bone
Os rostr.	=	rostral bone
Palat. mol.	=	soft palate
Phar.	=	pharynx
Plic. voc.	=	vocal fold
Proc. parac.	=	paracondylar process
Proc. voc.	=	vocal process of arytenoid cartilage
Proc. xiph.	=	xiphoid process
Pulm.	=	lungs
Scap.	=	scapula
Stern.	=	sternum
Sulc. med.	=	dorsomedian trench of soft palate
Thyroh.	=	thyrohyoid
Trach.	=	trachea
T1, T4	=	1st, 4th thoracic vertebra
Vest. lar.	=	laryngeal vestibule
Vest. nasi	=	nasal vestibule
VTL	=	vocal tract length
VVF(s) (dex.)	=	(right) velar vocal fold(s)

Results

Nostrils, nasal vestibulum and nasal cavity

The nostrils of both sexes are obliquely oriented, from laterodorsal to ventromedial in frontal view. In lateral view, starting from the nares, the narrow, tube-like nasal

vestibulum first courses 80° dorsally towards the nasal dorsum before it bends sharply in a caudal direction to connect to the larger volume of the nasal cavity proper. Caudally, the upward portion of the nasal vestibulum is supported by a small rostral bone (*Os rostrale*) at the rostral end of the nasal septum, resting mediosagittally on the incisive bone (Fig. 3, 8). From the most dorsal point of the nasal vestibulum, at eye level, the air passage runs slightly oblique caudoventrally (10°-15°) and almost straight, along the ventral nasal meatus and the nasopharyngeal meatus up to the choanae.

Choanae

The osseous choanae for both sexes are located comparatively far rostrally, at a level just caudal to the last molars (M^4/M_4). In lateral view, the lateral osseous laminae of the palatine and pterygoid bones have a very oblique ventral edge (caudally inclined by about 45°) as a foundation for anchoring the thick soft palate. As a consequence of the great dorsoventral height of the soft palate at this level, the nasopharyngeal meatus is narrow dorsoventrally and transversely and courses horizontally along the skull base, passing by the long pterygoids and the auditory bulla, before sharply bending ventrally towards the nasopharynx at a level between the auditory bulla and the paracondylar processes.

Cheek pouches

Cheek pouches are present in both the male and the female (S-Fig. 1). They are positioned immediately caudal to the upper lip and the mouth angle, between the nostril and the eye. Their opening is obliquely oriented, from the small upper canine rostrodorsally, to the first premolars P^3/P_3 caudoventrally, following the contour of the upper lip. The dimensions of the cheek pouches for the female and male were: rostrocaudal length ~28/~35, dorsoventral height ~20/~25 and transverse width

~15/~20 mm, respectively (S-Table 2). The caudoventral half of the cheek pouches contains a layer of buccal salivary glands on its lateral surface and is covered by the buccinator muscle.

Pharynx

The upper pharynx is very spacious and extensible (S-Fig. 2; S-Video 1). It connects the caudal ends of the nasal and oral cavities to the larynx and oesophagus. The resting position of the head is approximately at a right angle to the longitudinal axis of the body. This results in a pronounced bending of the upper pharynx, the oropharynx in particular, while the head is kept in its typical resting position. In contrast, neck extension, as observed in the initial phase of call production, will straighten the pharynx. Due to the short snout and the short hard palate, the choanae are positioned rather far rostrally, between the upper last molars (M^4), and about half way along the length of the head from a lateral view. The rostroventral, lateral and caudoventral parts of the pharyngeal wall receive terminating fibres from the genioglossolaryngeal muscle (see below and S-Tab. 7). The mucosa of the nasopharynx resembles the respiratory mucosa in the respiratory region of the nasal cavity. The nasopharyngeal mucosa is finely plicated, pliable and highly elastic. The mucosa of the oropharynx resembles the cutaneous mucosa of the oral cavity. The oropharyngeal mucosa is corrugated and also pliable and highly elastic. Internally, the oropharynx is completely separated from the nasopharynx, except for one connection just rostral to the laryngeal entrance, the intra-pharyngeal ostium (IPO). The soft palate is large, thick, and of triangular shape when viewed from a mediosagittal perspective. Thickness is maximal close to the choanae and tapers caudally towards the IPO. Inside the triangular space, between the roof of the oropharynx and the floor of the nasopharynx, the palatopharyngeal

muscle surrounds the IPO on both sides (S-Tab. 7). When the pharynx is manually extended (Fig. 4, 5) the IPO remains close to the epiglottis and the laryngeal entrance.

Soft palate

The overall dimensions of the soft palate in the female and male were: resting length – 50/70, extended length – 75/100 and dorsoventral thickness at the choanae 15/20 mm, respectively (S-Tab. 3). The entire soft palate is remarkable: its roof (= the mucosa of the dorsal surface of the soft palate) has a deep (~ 2 mm) median trench along its entire length in both sexes, although it is less prominent in the female. In the male, its transverse width in the resting position is only 1-2 mm, i.e. it is almost closed, whereas in the bilaterally extended state it is 6-8 mm wide, and becomes progressively wider towards the IPO. There is a median, serrated crest along the trench that terminates about 10 mm rostral to the IPO (Fig. 6A). The rostrocaudal length of the crest is ~ 20 mm and tapers rostrally. Maximum dorsoventral height of the crest is ~ 3 mm. Lateral to the median trench, the mucosa is flat and smooth and the two halves of these elevated surfaces of the floor of the nasopharynx are both ~ 5 mm wide (transversely). The surface of the median trench is covered with small knobs or caruncles. The lateral flat surfaces are a light whitish/greenish colour, whereas the mucosa of the median trench is dark reddish/brownish. The mucosa of the median trench is supplied with many tiny blood vessels.

In addition, the male and female IPO is not a simple opening between the dorsal and ventral compartments of the pharynx, as it is in other mammals (S-Fig. 3). Instead, its medial edges form two large, longitudinally oriented, voluminous folds, which protrude ventrally into the caudal part of the oropharynx (laryngopharynx) (Fig. 6B). These folds are termed ‘velar vocal folds’ (VVFs - Charlton et al 2013).

The mucosa of the ventral surface of the soft palate possesses a shallow median depression, about 9 mm wide rostrally, that narrows to 3 mm halfway along the rostrocaudal length. Along its caudal third, the depression widens to form a triangle that merges with a broad, caudally open horseshoe-shaped fold, which encompasses the velar vocal folds. Its longitudinal limbs, 6 mm wide rostrally, taper in caudal direction and fuse with the ventrocaudal parts of the velar vocal folds (VVFs) (Fig. 6B). The palatopharyngeal arc consists of a narrow fold surrounding the IPO, the VVFs, and the horseshoe-shaped fold, before fusing with the dorsocaudal wall of the pharynx caudal to the VVFs. On each side there is a rostrally closed blind space of about 15 mm in rostrocaudal length (destroyed on the left side when cutting into the pharynx), between the horseshoe-shaped fold medially and the palatopharyngeal arc laterally. The entire mucosa of the ventral surface of the soft palate is finely plicated transversely (Fig. 6B).

Velar vocal folds

VVFs occur in the male and the female. Rostral to the IPO, the lateral flat ventral surfaces of the soft palate rise up to form two longitudinally oriented, voluminous folds medially along the edges of the IPO (Fig. 6B). These velar vocal folds are clearly distinct from the surrounding soft palate. They narrow the IPO considerably, restricting it to a slit-like opening that is slightly wider rostrally than caudally. Laterally, the velar vocal folds are encompassed by a horseshoe-shaped fold and, further laterally, by the palatopharyngeal arc. Caudally, each VVF fuses with the respective limb of the horseshoe-shaped fold. The velar vocal folds protrude ventrally into the laryngopharynx (Fig. 3). The dimensions of the VVFs for female and male (n = 10, respectively) are: resting length - 26.2±2.59 versus 32.5±2.8, resting depth – 11.7±1 versus 14.9±2.13, resting width – 7.6±0.74 versus 10.7±1.32 and maximally extended length – 43.6±5.1 versus 51.4±6.29 mm. (S-Tab. 4).

Histological examination of the VVFs reveals that they are differentiated from the surrounding soft palate. Longitudinal and cross sections (5 µm thickness) show a three layered structure. The superficial thin layer consists of a cornifying stratified squamous epithelium. The intermediate layer comprises two sublayers: a subepithelial thin layer of collagenous and elastic fibres running mostly parallel to the surface of the VVFs; and a thicker layer of very loosely organised short and mostly single collagenous fibres, intermingled with few, mostly single elastic fibres. This sublayer contains numerous small, thin-walled vessels, most likely venules or lymphatic vessels and nerves. The deepest layer, constituting the base of the VVFs, is composed of a scaffolding of larger bundles of collagenous and elastic fibres, and striated muscle fibre bundles interspersed with groups of mucinous glands, larger blood vessels and nerves. The collagen and elastin fibres within this deep layer are mostly longitudinal in arrangement. The basal deep layer makes the transition to the larger muscle bundles, the larger, mostly transversely arranged, scaffolding of collagenous and elastic fibres, larger aggregations of mucinous glands, and larger blood vessels and nerves of the soft palate proper (Fig. 7).

Although the structure of the VVFs is broadly similar in males and females, there are a few key differences. The VVFs of the dissected male specimen are larger than those of the female (S-Tab. 4), and the second (b) sublayer of the intermediate layer appears to contain a higher overall density of collagenous fibres, which are organised in thicker bundles in the male than in the female. In the male there is also a large, longitudinally oriented bundle of elastic fibres, running parallel to the free edge of the VVFs in the intermediate layer. This bundle appears to be lacking in the female VVFs. The deep layer of the VVFs also appears to be more muscular in the male than in the female.

Hyoid apparatus

The koala's hyoid apparatus is suspended from the skull base by a pair of highly resilient hyoid ligaments. Each of these hyoid ligaments consists of several sub-ligaments that run parallel to one another. The dorsal attachment to the skull base is located between the auditory bulla and the paracondylar process. This attachment is supported by a small occipitohyoid muscle. Ventrally, the hyoid ligaments connect to the lateral ends of the ceratohyoids. Other parts of the suspension apparatus, for example, the tympanohyoids, stylohyoids, and epihyoids, are lacking. Interestingly, the hyoid ligaments of the male appear to be more elastic than those of the female: resting length versus maximally extended length in the female 30/60 mm,(100% extension) and 40/100 mm in the male, (150% extension) (S-Tab. 5). The ceratohyoids are short, cartilaginous, and fused rostrolaterally to the transversely oriented osseous basihyoid. Caudolaterally, the basihyoid connects to two thyrohyoids that are directed caudodorsally (S-Fig. 4). The larger rostral part of the thyrohyoids is ossified whereas the caudal ends are cartilaginous. The cartilaginous part of the thyrohyoids connects to the rostral horn of the thyroid cartilage (Fig. 8).

Thyrohyoid membrane

The thyrohyoid membrane extends between the thyroid cartilage and the thyrohyoid laterally, and the thyroid cartilage and the basihyoid ventrally. It is short and rather tough, with a ventral length that exceeds its lateral length. Rostrocaudal resting length for the female and the male are 2-3/3-4 mm laterally and 6-7/10-11 mm ventrally; maximally extended lengths are ~4/~5 mm laterally and 8-9/12-13 mm ventrally (S-Tab. 6). Due to the stiffness of the thyrohyoid membrane, when the larynx is manually retracted the ventral parts of the hyoid apparatus (ceratohyoids, basihyoid, thyrohyoids) stay close to the larynx as it descends down the neck towards the thorax. The highly elastic hyoid ligament permits the larynx to be fully retracted into the thoracic

inlet. The laryngeal entrance and the ventral parts of the hyoid apparatus are then located at the level of the manubrium.

Musculature

The results of the ventral neck musculature dissection, including presumed muscle functions, are summarized in S-Table 7. Instances where muscles deviate greatly from the typical mammalian pattern are shortly listed below:

1) Three muscles, the digastric, the omohyoid and the sternohyoid, share a common tendinous intersection. In the koala the omohyoid and sternohyoid muscles have entirely lost their connection to the hyoid apparatus (Fig. 9).
2) The caudal fibres of the genioglossus muscle are of considerable length and do not terminate on the basihyoid. Instead they terminate on the lateral wall of the oropharynx and on the thyrohyoid membrane close to the rostral edge of the thyroid cartilage. As a consequence of this, it is henceforth called a genioglossolaryngeal muscle (Fig. 5).
3) The geniohyoid muscle is greatly extended in length and bunches up to form undulating reserve bends during the resting state of the larynx. It passes through a guiding arch provided by the hyoglossus muscle (S-Fig. 5).
4) The sternohyoid and sternothyroid muscles are deeply anchored in the thorax. The truly intrathoracic origins of both muscles arise from the dorsal surface of the sternum and the first few costal cartilages (Fig. 10).
5) The mylohyoideus and hyoglossus muscles have completely lost their attachment to the hyoid apparatus. In the koala the hyoglossus muscle forms a type of guiding arch for the long geniohyoid muscle (S-Fig. 5).
6) The stylohyoid, caudal stylopharyngeus, and hyopharyngeus muscles are difficult to compare with the corresponding muscles of placental mammals

because an osseous suspension of the hyoid apparatus to the skull is lacking in the koala. MacAlister (1872) and Young (1882) realised this and combined them as the styloid group. We found that the main portion of this muscle sheet originates from the thyrohyoid, and terminates on the base of the skull, caudally adjacent to the attachment of the hyoid ligament. We therefore suggest that these three muscles are grouped tougher under a new name: the hyocephalic muscle (Fig. 11).

Larynx

Larynx dimensions

Overall measurements of the larynx for female (n = 9) and male (n = 10) koalas are: maximal rostrocaudal length 25.2±1.66 versus 28.2±2.68 mm, maximal transverse width 22.0±2.13 versus 25.2±2.68 mm and maximal dorsoventral height 16.7±0.78 versus 19.5± 1.62 mm (S-Table 8).

Laryngeal cartilages

In both sexes, the cartilages of the larynx comprise the epiglottis, the thyroid cartilage, the cricoid cartilage, the two arytenoid cartilages, the proarytenoid cartilage, resting on the rostral commissure of the medial processes of the arytenoid cartilages, and a small postarytenoid cartilage located caudal to the commissure of the medial processes of the arytenoid cartilages (Fig. 12). The thyroid and cricoid cartilages are fused ventromedially (as previously noted, e.g., by Symington 1898; Schneider 1964 - S-Fig. 6). The vocal process of the arytenoid cartilage is crescent-shaped, rostrally directed, and protrudes considerably in a ventral direction towards the laryngeal lumen. The vocal process extends far rostrally, up to the level of the proarytenoid cartilage and approaching the laryngeal entrance (Fig. 12). On either side of the cricoid, the paired

518 dorsal cricothyroid connection is established by a synchondrosis, which is located
519 dorsal to the caudal laryngeal nerve. Laterally, the proarytenoid, the arytenoids, the
520 postarytenoid, and the cricoid cartilage(s) are covered by the thyroid lamina and the
521 broad rostral and long caudal horns of the thyroid cartilage.

522 The epiglottis is spatula-shaped in dorsal view. Medioventrally, its base
523 connects to the rostral edge of the thyroid cartilage. Its laryngeal surface faces the
524 laryngeal vestibule whereas the ventral third of its lingual surface serves as the origin
525 of the hyoepiglottic muscle. The dimensions of the epiglottis for female (n = 8) and
526 male (n = 9) koalas were: rostrocaudal length 10.1±0.92 versus 11.0±0.77 mm and
527 transverse width 12.2±1.08 mm versus 14.8±1.85 mm (S-Table 9). The colour of the
528 epiglottic cartilage is yellow, suggesting a composition of elastic cartilage (Fig. 12A).
529 Cuneiform processes are lacking in both sexes.

530 The thyroid cartilage has a short, ventrally-curved rostral horn that establishes
531 the cartilaginous connection to the thyrohyoid of the hyoid apparatus. In contrast, the
532 caudal horn is long and straight, and protrudes caudally in parallel to the longitudinal
533 axis of the larynx. The laminae of the thyroid cartilage are of trapezoid shape in lateral
534 view, their ventral rostrocaudal length exceeding the dorsal by about one third. A large
535 fat pad is sandwiched between the thyroid lamina laterally and the thyroarytenoid and
536 ceratocricoarytenoid muscles medially. A thyroid bulla is not observed in the koala.

537 The cricoid cartilage has almost the same rostrocaudal dimensions dorsally and
538 ventrally, i.e. the dorsal cricoid lamina is very short. In the dorsal third, the left and right
539 rostral edges of the cricoid cartilage bulge out rostrally and form the articular surfaces
540 for connection with the arytenoid cartilages. Together with the ventrally and dorsally
541 fixed connections to the thyroid cartilage, the bilateral bulging creates a boomerang-
542 shaped opening between the rigidly coupled thyroid and cricoid cartilages, its convex

shape being directed rostrally. In lateral view, the caudal edge of the cricoid cartilage, to which the first tracheal cartilage connects, is of concave shape.

The arytenoid cartilages are comparatively large and articulate with the dorsorostral bulges of the cricoid cartilage. Rostrally, the arytenoid cartilages extend upwards to form the caudal boundary of the laryngeal entrance. Each arytenoid cartilage possesses three well-developed processes: a lateral muscular process, a (dorso)medial process, and a ventrally directed vocal process. Corniculate processes of the arytenoid cartilages are lacking, and left and right arytenoid cartilage are linked to each other via their medial processes by connective tissue. The dorsoventral extent of the arytenoid cartilage is considerable. The vocal process protrudes widely in a ventral direction, and causes a slight prominence of the laryngeal mucosa at its ventral tip, to which the dorsal end of the vocal fold attaches.

The proarytenoid cartilage, which is also termed procricoid cartilage in the older literature, is a homologue of the interarytenoid cartilage in placental mammals (Symington 1898; Schneider 1964, p. 42). It is located rostral to where the medial processes of the arytenoid cartilages connect and its caudal surface is intimately linked to this transverse junction by connective tissue.

Intrinsic laryngeal muscles

The cricothyroid muscle is lacking, possibly as a result of the ventral fusion of the thyroid and cricoid cartilages, and the concomitant restriction in mobility between these two cartilages. The thyroarytenoid muscle is a homologue of the corresponding muscle of placental mammals (Schneider 1964, p. 34). It originates medioventrally from the thyroid lamina and its fibres course laterodorsally, before terminating on the muscular process of the arytenoid cartilage ventrally. The thyroarytenoid muscle does not connect to the vocal process of the arytenoid cartilage.

The lateral cricoarytenoid muscle is also a homologue of the corresponding muscle of placental mammals (Schneider 1964, p. 34). It originates from the laterodorsal surface of the cricoid cartilage, where it is partly covered by the ceratocricoarytenoid muscle. Its fibres course obliquely rostrodorsally and terminate lateroventrally on the muscular process of the arytenoid cartilage.

The aryprocricoid muscle in the koala is a homologue of the transverse arytenoid muscle of placental mammals (Schneider 1964, p. 35). It originates from the arcuate crest of the arytenoid cartilage and most of its fibres terminate on the proarytenoid cartilage, whereas the most rostral fibres fuse with those of the contralateral muscle.

The cricoprocricoarytenoid muscle originates from the dorsal edge of the cricoid cartilage and terminates laterocaudally on the procricoid cartilage.

The koala's ceratocricoarytenoid muscle is a homologue of the dorsal cricoarytenoid muscle of placental mammals (Schneider 1964, p. 44). It originates from the medial surface of the caudal horn of the thyroid cartilage, the lateral surface of the cricoid cartilage, and the laterodorsal parts of the cricoid lamina. The muscle terminates dorsally on the muscular process and the arcuate crest of the arytenoid cartilage. We assume that the function of the ceratocricoarytenoid muscle is to dilate the glottis.

Laryngeal entrance and cavity

The laryngeal entrance is surrounded by the epiglottis rostrally, the aryepiglottic folds laterally, and the arytenoid cartilages caudally. A large pad of small glands is laterally attached to each of the aryepiglottic folds, and mostly covered by the thyroid laminae. The laryngeal cavity comprises a vestibule extending from the entrance of the larynx to the vocal folds, the glottis between the vocal folds, and an infraglottic space, extending from the vocal folds to the exit of the larynx at its junction with the trachea.

As a consequence of the large vocal process of the arytenoid cartilages and the relatively short vocal folds, the ratio of the intercartilaginous to intermembraneous portions of the glottis is approximately 50:50.

Laryngeal vocal folds

The laryngeal vocal folds (LVFs) are rostrally inclined against the longitudinal axis of the larynx by about 30°, from craniodorsally to caudoventrally. Ventrally, the LVFs attach to the dorsal surface of the fused thyroid and cricoid cartilages, close to the cranial edge of the cricoid arch in a paramedian plane. Dorsally, the LVFs attach to the tip of the vocal process of the arytenoid cartilage. The dimensions of the LVFs for female (n = 9) and male koalas (n = 10) respectively are: dorsoventral resting length 8.0±0.57 versus 9.8±0.88mm, rostrocaudal resting length 0.8±0.1 versus 1.0±0.13 mm, transverse resting width 0.5±0.12 versus 0.8±0.16 mm and maximally extended dorsoventral length 9.8±0.39 versus 11.8±0.94 mm (S-Tab. 10).

Laryngeal mobility

The resting position of the larynx is approximately at the level of cervical vertebrae 4-6 in an adult male koala (Fig. 8). The larynx can be manually retracted down into the thoracic inlet in both sexes. At maximal retraction, the ceratohyoids and the basihyoid are close to the level of the first rib, and the maximally extended hyoid ligament runs more or less parallel to the ventral edge of the hypaxial neck musculature. Larynx movements down and up the ventral neck region are facilitated by a highly flexible double gliding layer of connective tissue that dorsally separates the pharynx, larynx and oesophagus from the hypaxial neck musculature (longus capitis, longus colli muscles) (S-Fig. 7). The medial retropharyngeal lymph nodes are located on the dorsal wall of the pharynx at the level of the first tracheal rings, contacting the dorsal gliding

layer. The lateral retropharyngeal lymph nodes are located a bit more cranially, at the level of the osseous hyoid apparatus in its resting position, and in the groove between the nasopharynx and the neck musculature. Ventrally, the muscular channel or guidance for any potential craniocaudal movements of the pharynx, larynx and oesophagus, provided by the inter-linked digastric, omohyoid and sternohyoid muscles, is lined with a connective tissue layer. In addition, there is a fat cushion between the styloglossus muscle laterally and the genioglossolaryngeal muscle medially, and a short connective tissue canal at the thoracic inlet, through which the larynx can move. This canal is attached to the clavicula, the carotid artery, the neck fascia and the sternal manubrium. This gliding space continues inside the thorax, dorsal to the heart and the large blood vessels at the base of the heart, which are both covered by the pericardial pleura. Movement of the larynx, oesophagus and trachea inside the thorax is further guided by the thoracic portions of the hypaxial neck musculature (longus colli muscle) dorsally and the sternohyoid and sternothyroid muscles ventrally. A gliding space for minor cranio-caudal movements of the intrathoracic portions of the sternohyoid and sternothyroid muscles along the craniodorsal surface of the sternum is provided by the common connective tissue sheath interlinking these muscles, ventrally, and by the dorsally adjacent pericardial pleura.

Vocal tract length

Oral vocal tract length (hereafter VTL) was taken as the distance from the lips to the LVFs, and the nasal VTL was measured from the tip of the nostrils to the LVFs. The dimensions of the koala's nasal and oral vocal tract lengths during different stages of the dissection of one female and one male koala are: resting nasal vtl with angled head

160-165 versus 185-190 mm, maximally extended nasal vtl with angled 185-190 versus 230-240, maximally extended nasal vtl with head and neck extended (male only 255-260 mm; resting oral vtl with angled head 140-145 versus 160-170 mm, maximally extended oral vtl with head and neck extended (male only) 210-220 mm (S-Tab. 11). The laryngeal vocal folds are not involved in the production of male koala bellow vocalizations, and it is presumed that the glottis is open during call production (Charlton et al 2013). Accordingly, we also provide additional measurements of vtl from the nostrils and lips to the VVFs (a) and from the nostrils and lips down to the tracheal bifurcation (b) at maximal extension in the male specimen: nasal vtl ~220 mm (a) and ~ 295 mm (b), oral vtl ~190 mm (a) and ~270 mm (b) (S-Tab. 11).

Nerves

The nerve supply to the pharynx, larynx, and associated muscles did not appear to differ between the male and female adult koala specimens. The hypoglossal nerve emerges from the skull base ventral to the external acoustic meatus, between the auditory bulla rostrally and the paracondylar process caudally. This nerve curves laterally around the hyoid ligament obliquely in a rostral direction, and along the ventral edge of the styloglossus muscle. It then enters the hyoglossus muscle laterally before subdividing into 4 branches: three branches curve dorsally along the rostral edge of this muscle to innervate the tongue and supply the intrinsic tongue muscles. The most ventral of these branches supplies the rostral part of the genioglossolaryngeal muscle. This branch is undulating, while the dorsal two are not. The most ventral branch of the hypoglossal nerve is also an undulating nerve that supplies the geniohyoid muscle.

Motor nerve supply to the omohyoid, sternohyoid and sternothyroid muscles is provided by the ventral ramus of the first cervical nerve (N. cervicalis I). As a

consequence of the long sternohyoid and sternothyroid muscles, the associated nerve fibres are of corresponding length. The lengths of the more or less straight branches to the caudal, intrathoracic parts of the sternohyoid and sternothyroid muscles are around 150 mm in the female and around 200 mm in the male. In contrast, branches to the cranial parts of the sternohyoid and sternothyroid muscles form caudally convex reserve loops that are slightly undulating.

The glossopharyngeal nerve emerges from the skull base close to the paracondylar process. It traverses the caudal stylopharyngeal muscle in a rostral direction and provides a small branch to this muscle. The pharyngeal branches of the glossopharyngeal nerve to the dorsal wall of the pharynx were not dissected. The larger lingual branch of the glossopharyngeal nerve runs ventrally along the rostral border of the caudal stylopharyngeal muscle and medial to the hyoid ligament before sharply bending rostrally towards the lateral wall of the oropharynx and the tongue. On the way it subdivides into a dorsal branch leading to the soft palate and a ventral branch leading to the root of the tongue. All of these branches of the glossopharyngeal nerve are strongly undulating nerves.

The vagus nerve emerges from the skull base together with the glossopharyngeal nerve. The oesophageal branch of the vagus nerve is a strongly undulating nerve and courses caudally along the nasopharynx towards the caudal constrictor muscles of the pharynx and the oesophagus. At the level of the thyroid cartilage it subdivides into three branches supplying the thyro- and cricopharyngeal muscles and the vestibulum of the oesophagus (Fig. 13). A further branch of the vagus nerve, the cranial laryngeal nerve, is also strongly undulating. It courses caudally along the caudal edge of the hyocephalic muscle and pierces the thyropharyngeal muscle before entering the larynx with its ventral branch. The strongly undulating lingual

691 branch of the glossopharyngeal nerve, the cranial laryngeal nerve, and the
692 oesophageal branch of the vagus nerve appear to be enclosed in hose-like connective
693 tissue sheaths (Fig. 13).

694 Trachea

695 In both sexes all the tracheal cartilages between the cricoid cartilage and the bronchial
696 cartilages are dorsally separated by a ~ 5 mm wide gap that is bridged by elastic
697 connective tissue i.e. they do not touch dorsally. This membranous wall continues
698 dorsally onto the main bronchi. The number of tracheal cartilages (from cricoid to
699 bifurcation) was 17 in the female and 18 in the male. Both the resting and the maximally
700 extended lengths of the trachea did not differ appreciably between male and female: ~
701 40 mm and ~ 70 mm, respectively.

702 Sexual dimorphism of vocal anatomy and acoustic values

703 In order to evaluate the measured differences of vocal features and acoustic values
704 between males and females, intersexual body size differences have to be considered
705 (Tab. 1)

706

707 **Discussion**

708 This study provides the first comprehensive description of the koala's vocal anatomy.
709 Although anatomical peculiarities of the vocal organs and the associated musculature
710 in koalas were noted by the classical morphologists (MacAlister 1872; Young 1882;
711 Symington 1898; Sonntag 1921), they were mostly considered in a taxonomic context.
712 By combining detailed anatomical data obtained using modern imaging and
713 histological techniques, systematic dissections, behavioural observations, and

published acoustic data, we are now able to provide a comprehensive and functional account of the koala's unusual vocal anatomy.

A simple, but descended larynx and a specialised hyoid apparatus

Although most features of the koala larynx correspond to those of other marsupials, a rostral thyroid bulla, generally present in marsupials, is lacking in the koala (Schneider 1964, p. 9). One of the key differences between the marsupial larynx and the larynx of placental mammals is the characteristic ventral fusion of the thyroid and cricoid cartilages observed in the former. This longitudinal cartilaginous connection is derived and arises postnatally (Symington 1898; Schneider 1964, p. 8, 14). The greatly restricted mobility between both cartilages is likely to explain why the marsupial larynx lacks a cricothyroid muscle (Schneider 1964, p. 26). In placental mammals, the mutual mobility of thyroid and cricoid cartilages and coordinated activities of the thyroarytenoid and cricothyroid muscles represent a major means of tensioning the laryngeal vocal folds (LVFs), thereby increasing the range of producible fundamental frequencies (F0) (Titze 1993; Titze et al 2016). As a consequence of the stiffened thyroid/cricoid connection and the lacking cricothyroid muscle in the marsupial larynx, the potential F0 range and control over F0 is either reduced or differently achieved in marsupials.

The 50:50 ratio between the intercartilaginous and intermembraneous portions of the koala glottis indicates a large vocal process of the arytenoid cartilage, which widely protrudes rostroventrally along the inner laryngeal wall (Schneider 1964, p. 17). In our specimens we found that male LVFs were ~ 10 mm and female LVFs were ~ 8 mm long. Considering the LVFs and their predicted F0 frequency of ~ 400 Hz (Charlton et al 2013), the LVFs are probably only involved in the production of the higher F0 vocalizations produced by this species. In addition, both male and female koalas the LVFs are rostrally inclined, and dorsally attach to the rostrally directed vocal process.

739 A comparable rostral inclination occurs in wild boar (*Sus scrofa*); however, in this
740 placental species the vocal process is caudally directed (Schneider 1964, p. 64f, fig.
741 66). The koala larynx does not possess lateral laryngeal ventricles and, as a result,
742 vestibular folds are also lacking (Fig. 12). The lack of a thyroid bulla and lateral
743 laryngeal ventricles means that there are virtually no intra-laryngeal resonance spaces.

744 Interestingly, in the koala, all the dorsal suspensory elements of the hyoid
745 apparatus (except the ceratohyoids) have been evolutionarily transformed into a highly
746 resilient hyoid ligament. Due to the short cartilaginous connection between the
747 thyrohyoid and the larynx and the short and tough thyrohyoid membrane, it is likely that
748 the ventral cartilaginous and osseous parts of the hyoid apparatus, i.e. the
749 ceratohyoids, the basihyoid and the thyrohyoids, are retracted together with the larynx
750 towards the thoracic inlet during call production (see 'Laryngeal mobility, calling
751 posture and the VVFs'). Because the sternohyoid muscles have lost their attachment
752 to the hyoid apparatus, they cannot support the sternothyroid muscles in retracting the
753 ventral parts of the hyoid apparatus and larynx towards the sternum (see 'Muscles and
754 nerves of the vocal tract').

755 A novel organ for low frequency production

756 The most spectacular evolutionary transformation of the koala's vocal organs has
757 occurred in the region of the intra-pharyngeal ostium (IPO). In both sexes, the lateral
758 edges of the IPO have been transformed into two large, longitudinally oriented,
759 voluminous folds that protrude ventrally towards the laryngeal entrance and medially
760 towards the midline (cf. McCurry et al 2016 Fig. 6; S-Fig. 3). These extra laryngeal
761 folds have previously been termed 'velar vocal folds' or VVFs because they look
762 remarkably similar to large laryngeal vocal folds (Charlton et al 2013). This previous
763 study also confirmed that VVFs function as extra-laryngeal acoustic sources in male

764 koalas (Charlton et al 2013), allowing them to produce the extremely low F0 of bellow
765 vocalisations (circa 27 Hz). While we confirm here that female koalas also possess
766 VVFs, in our specimens the male has larger VVFs (Tab. 5), which protrude further into
767 the laryngopharynx than in the female, indicating that these structures are significantly
768 more developed in males than in females.

769 Owing to their position and orientation, the VVFs can only function as a sound
770 source during nasal inhalation phases of vocal production. This function requires
771 tensioning and closure of the IPO, which can be described as a 'velar glottis'. When
772 we extended the pharynx in our specimens the IPO remained close to the laryngeal
773 entrance, confirming that the VVFs are ideally located for sound production during
774 nasal inhalations. The VVFs are also heavily wrinkled in their resting (relaxed) state,
775 which might provide the reserve tissue necessary for allowing the dramatic length
776 changes accompanying larynx retraction and tensioning of the soft palate, including
777 the VVFs and the closure of the IPO prior to vocal production. Functionally, this would
778 correspond to the tensioning and bringing together of the LVFs by muscle-induced
779 movements of the arytenoid cartilages inside the larynx.

780 It is also worth noting that narrowing of the IPO by the VVFs is expected to
781 impede nasal respiration. Therefore, except prior to vocal production, the VVFs should
782 narrow the IPO as little as possible. As the VVFs cannot be pivoted laterally by
783 cartilage-muscle–induced pulling, the change between respiratory and phonatory
784 position must be achieved by a different mechanism. Shrinking of the VVFs after vocal
785 production might be facilitated by their distinct wrinkles in the resting position.
786 Additionally, expansion of the dorsal trench and the longitudinal plication of the soft
787 palate, and relaxation of the palatopharyngeal muscle (the constrictor of the IPO) might
788 be involved in keeping the IPO open. These features may also play a role in bellow-

synchronous pharynx expansion (S-Fig. 2; S-Video 1). Opening and closing of the IPO might also be regulated by coordinated contractions of the strap muscles (inducing length changes) and palatopharyngeal muscle (inducing width changes).

Our histological examination reveals that the VVFs are organised into three discrete layers, approximating the structure of placental mammal LVFs (Hirano et al 1981; Kurita et al 1983; Hirano & Kakita 1985), although on a larger scale. The structure of the VVFs can be interpreted as a body-cover complex, in which the third, deep layer (muscle bundles and connective tissue scaffolding) represents the 'body' and the upper two layers (epithelium and sub-epithelial loosely organised connective tissue) the cover. Hence, the VVFs consist of a robust basal region near their transition to the soft palate and a soft and deformable apical region close to their free edge flanking the IPO. Accordingly, the VVFs, like the LVFs, can be described as a layer-structured oscillator (Hirano & Kakita 1985).

In addition, we found that male VVFs contain more collagen fibres than female VVFs, and have a large bundle of elastin fibres in the lower portion of the second layer of the VVFs. In humans, significantly higher levels of collagen are found in the male vocal fold (Chan et al 2007) and testosterone is known to increase the collagen/elastin ratio of tissue (Fischer & Swain 1980). If we consider that the levels of collagen and elastin could contribute differentially to VVF elasticity, testosterone exposure during development, or the dramatic increase in testosterone that is known to occur in male koalas just prior to the breeding season (Mitchell 1990; Allen 2010), could facilitate low frequency, periodic oscillation of the VVFs during call production by increasing the stiffness of these structures, allowing males to produce their extremely low F0 bellow vocalisations.

Muscles and nerves of the vocal tract

814 Several muscles of the koala's throat and ventral neck region deviate considerably
815 from the typical mammalian pattern (cf. Nickel et al 1979). Although this has been
816 noted previously, to our knowledge, no functional explanation has been proposed so
817 far for this unique arrangement in the koala (MacAlister 1872; Young 1882; Sonntag
818 1921, 1922). Recent findings (Charlton et al 2013), along with observations of vocal
819 behaviour, now allow us to propose a functional explanation. We suggest that the
820 unusual, muscular organisation of the koala's throat and ventral neck region facilitates
821 the dynamic movement of the larynx-hyoid complex during production of bellow
822 vocalisations. The main retractor of the larynx and the ventral hyoid parts, the strap-
823 like sternothyroid muscle, has elongated and extended its origin deep into the thorax.
824 Pronounced contractions of this muscle would produce a strong pull on the larynx and
825 ventral hyoid parts, resulting in the caudal movement of these structures down into the
826 thorax. In the koala, unlike other mammals, the caudal movement of the larynx-hyoid
827 complex is not restricted by connections to the tongue and lower jaw because the
828 mylohyoid and hyoglossus muscles are not attached to the hyoid apparatus, and
829 because the genioglossolaryngeus and geniohyoid muscles have been greatly
830 elongated (Fig. 14).

831 We suggest that the return of the larynx and ventral hyoid parts to their resting
832 position is achieved by the resilience of the hyoid ligament and of the pharynx, which
833 become heavily extended during larynx and ventral hyoid retraction, and the constrictor
834 muscles of the pharynx, the hyocephalic muscle, and strongly elongated extrinsic
835 tongue muscles. Concomitant shortening of the pharyngeal walls would be supported
836 by contractions of the middle and caudal pharyngeal constrictors and the hyocephalic
837 muscle, all of which are elongated during the extension phase. The
838 genioglossolaryngeus muscle, which extends from the tongue down to the thyrohyoid

membrane and the rostral edge of the thyroid cartilage, would then be able to contract and return the larynx to its resting position (around the 4th cervical vertebra). The prominent, and greatly extended strap-like geniohyoid muscle, would additionally support the mobility of the larynx and ventral hyoid parts down and up the ventral neck region. For example, during the retraction phase, i.e. during contraction of the sternothyroid muscle, the undulations of the geniohyoid muscle will be stretched and contraction of the geniohyoid muscle during the protraction phase will restore the undulations. In addition, because the fibres of the hyoglossus muscle diverge and connect to the tendinous intersection between omohyoid, sternohyoid and digastric muscle, it forms an arch around the long geniohyoid muscle. This arrangement ensures, analogous to the retinacula of certain tendons of limb muscles (cf. Clavero et al 2005; Numkarunarunrote et al 2007; Robertson et al 2007), that the geniohyoid muscle is kept in the correct position as the larynx is maximally retracted and then returned to its resting position. Protraction of the larynx and ventral hyoid parts will further be assisted by contraction of the hyocephalic muscle, which suspends these elements from the skull base (Fig. 14).

The three muscles that are connected to each other by a tendinous intersection (digastric, omohyoid and sternohyoid muscles), together with their contralateral counterparts, appear to function as a longitudinal muscular guiding channel, along which the pharynx, the ventral hyoid parts, and the larynx and trachea are able to glide in a rostrocaudal direction during their dynamic down and up movements. As the koala's omohyoid and sternohyoid muscles have lost their connection to the hyoid apparatus, they would not be involved in moving the ventral hyoid parts. In contrast, the sternohyoid muscle is likely to be very effective as a lateral guide bar because it extends dorsally from the sternum and costal cartilages up to the rostral portion of the

digastric muscle. Strong extension of the head and neck, as observed during bellow production, probably facilitates this guiding by stabilising, tensioning, and extending the channel along a straight line from the angle of the lower jaw down into the thorax. Rostrocaudal gliding movements of the ventral hyoid parts and the larynx are further facilitated by the double connective tissue layer intercalated between the hypaxial neck musculature and the cervical vertebrae dorsally and the pharynx, larynx and oesophagus ventrally (S-Fig. 7). In addition, because the digastric muscles terminate on the lower jaw, they are likely to act as an anchor for the guiding channel. However, in contrast to laryngeal movements in ruminants with a descended larynx (Frey et al 2008; 2011; 2012), the short and weak thyrohyoid muscle of koalas cannot assist in returning the larynx to its resting position because the ventral parts of the hyoid apparatus, including the thyrohyoid, descend together with the larynx during the retraction phase. Hence, the most parsimonious explanation for the profound evolutionary transformation of the koala's throat and ventral neck musculature is that it facilitates the retraction and return of the larynx and ventral parts of the hyoid apparatus during the production of this species' bellow vocalisations. The phylogenetic distance between koalas and ruminants (marsupial vs. placental species) suggests independent evolution of a descended and mobile larynx and the respective muscular organisation of these species reinforce this notion.

As the muscles involved in extensive rostrocaudal excursions of the larynx elongated and underwent considerable length changes during protraction and retraction of the larynx over evolutionary time, the nerves supplying these muscles had to elongate correspondingly. In order to accommodate the length changes of their target muscles these nerves evolved two different modes of adjusting to shortly and profoundly changing muscle lengths. First, the nerves to the rostral portions of the

889 ribbon-like strap muscles (M. sternohyoideus, M. sternothyroideus) evolved large,
890 caudally sagging loops, which can be stretched during muscle extension (e.g. by head
891 and neck extension, or by strong muscle contraction during larynx retraction). Second,
892 the nerves to the muscles in the region of the pharynx evolved strongly undulating
893 branches, which can also be stretched during extensions of the pharynx. Additionally,
894 the strongly undulating nerve branches evolved hose-like connective tissue sheaths to
895 facilitate stretching and rewinding of the nerves (Fig. 13). Similar undulating nerves
896 have been documented in ruminants with a descended and mobile larynx, e.g. Iberian
897 red deer (*Cervus elaphus hispanicus*) and goitred gazelle (*Gazella subgutturosa*) (Frey
898 et al 2011, 2012).

899 Laryngeal mobility, calling posture and the VVFs

900 In ruminant species with a descended and mobile larynx (e.g. red deer, fallow deer,
901 Mongolian gazelle, goitred gazelle) the larynx is retracted, thereby elongating the
902 pharynx (the acoustic filter), and the LVFs (the acoustic source) produce the
903 fundamental frequency at exhalation. In koalas the low fundamental frequency is
904 produced by the pharyngeal VVFs, and hence, laryngeal retraction must have a
905 different function.

906 In species that produce low fundamental frequencies using the LVFs, e.g.
907 elephants (Herbst et al 2012) sound production requires adduction of the arytenoid
908 cartilages, i.e. closure of the glottis, and tensioning of the LVFs by means of intrinsic
909 laryngeal muscle contractions. Then, and only then, can sustained periodic oscillations
910 of the LVFs be achieved by an air stream (typically produced by exhalation from the
911 lungs). While the structure of the VVFs clearly differs from that of the LVFs, the glottis-
912 like cleft in between these novel structures also has to be closed, and tension applied
913 to the VVFs before periodic oscillations can be induced (by an inhalatory airstream in

koala bellows (Charlton et al 2011b, 2013). Since the VVFs are not directly connected to any skeletal parts, closure of the 'velar glottis' and tensioning of the VVFs must be achieved in a quite different way from that of the LVFs.

The koala's VVFs are part of the pharynx. Therefore, to produce a bellow vocalisation both vocal tract elongation and tension on the VVFs have to be achieved simultaneously by appropriate adjustment of the pharynx. The lowering of the formants is produced by larynx retraction (as in ruminants with a descended and mobile larynx, for example: Frey et al 2008, 2011, 2012). In contrast to these ruminants, however (see above), the tension on the VVFs that is required to produce a source signal is effected by the same action. Strong extension of the pharynx will tend to bring the median edges of the VVFs together, thereby closing the 'velar glottis'. Simultaneously, strong extension of the pharynx, including the soft palate, will set the VVFs under tension. As such, laryngeal retraction leading to the extension of the pharynx seems to be the most likely mechanism for producing low F0 and formants in the koala (Charlton et al 2013). As a consequence of this coupled mode of sound production and formant lowering, and in contrast to ruminants with a descended mobile larynx and an intra-laryngeal sound source, production of the lowest frequencies in koalas requires maximal vocal tract extension, i.e. maximal extension of the acoustic filter. At low pharynx tension, the 'velar glottis' would not close and the VVFs would not be sufficiently tensed for producing the very low extra-laryngeal source frequencies.

As in male ruminants with a descended larynx, the extension of the pharynx in koalas is supported by a characteristic calling posture involving pronounced head and neck extension (Fig. 1 – for ruminants cf. Frey et al 2008, 2011, 2012). However, in contrast to ruminants, male koalas produce their mating calls in a characteristic two-stage posture, gradually changing from pronounced extension (position 1) to distinctive

939 flexion (position 2) of the head and neck region (Charlton et al, 2011). In the first
940 position, the neck is extended and the head turned upward so that the longitudinal axis
941 of the head is held in parallel to the longitudinal axis of the body (Fig. 1A). In the course
942 of the call, along with numerous vocal in- and exhalations, the head is successively
943 lowered until, as the very low F0 and formants are produced at the end of the call, it
944 reaches the second position (Fig. 1B). Here, the longitudinal axis of the head is either
945 held at an obtuse angle relative to the longitudinal axis of the body, or the chin is pulled
946 a bit more ventrally towards the sternum (Charlton 2011b). Presumably, position 1
947 involves pronounced larynx retraction down to the thoracic inlet, thereby effecting
948 longitudinal extension of the pharynx. In position 2, the angular position of the head
949 will raise the base of the skull, to which the rostral portion of the pharynx is attached,
950 thereby achieving the utmost of pharynx extension. During previous experiments on
951 koala cadavers we found that position 2 was best for setting the VVFs under
952 appropriate tension to produce this species' characteristically low F0 (Charlton et al
953 2013).

954 Sexual dimorphism of vocal anatomy versus acoustics

955 Using head length as a proxy for overall body size, male Queensland koalas are
956 around 9% larger than females (Ellis & Bercovitch 2011). In the current study we found
957 that male head length (measured from the occipital ridge to the tip of the nasal bone:
958 Charlton et al 2011) was around 10% greater than female head length (Tab. 1), which
959 accords well with the findings of Ellis and Bercovitch on 35 male and female koalas
960 (2011), and suggests that our relatively small sample of six males and females was
961 representative of the Queensland koala. Male body length was around 4% greater
962 than female body length in our sample (Tab. 1). Accordingly, if we assume that male

963 koalas are between 4-10% larger than females, then the size dimorphism in vocal
964 anatomy is greater than expected (Tab. 1).

965 When we consider how size dimorphism in vocal anatomy relates to acoustic
966 differences between male and female koalas, we find that the 15-16% longer male
967 vocal tract corresponds extremely well to the documented 16% lower ΔF of male
968 koalas when compared to females (Tab. 1) (Charlton et al 2011, Charlton 2015). Since
969 differences in male and female vocal tract length closely track differences in male and
970 female formant spacing, these findings lend further support to the notion that the
971 broadband frequency components that have been identified in previous studies
972 (Charlton et al 2011, Charlton 2015) are indeed formants. The production of the
973 extremely low formant frequencies in male and female bellows remain unexplained.
974 We suggest that koalas use the VVFs to simultaneously excite resonances in the oral
975 and nasal vocal tract, including “sub-laryngeal” spaces like the trachea and the main
976 bronchi. Future studies that use precise geometrical data to predict the centre
977 frequencies of the oral and nasal vocal tracts (including the sub-laryngeal air spaces)
978 are now required to confirm how koalas produce such low formant frequencies for their
979 size.

980 Because koalas produce the exceptionally low F0 of bellow vocalisations using
981 their VVFs (Charlton et al 2013), we would also expect bellow F0 dimorphism to track
982 VVF dimorphism. We found that male VVFs were 24% longer than female VVFs, yet
983 male bellow mean F0 (of 28.7 Hz) is only 8% lower than the female bellow mean F0
984 (of 31.3 Hz) (Tab. 1). However, much closer correspondence between VVF sexual
985 size dimorphism and differences in male and female minimum bellow F0 was found
986 (Tab. 1), illustrating that the minimum producible F0 is ultimately constrained by the
987 length of the oscillating structure generating the sound (in this case the VVFs).

Interestingly, male LVFs are 22% longer than females, which is far above the ~4-10% size difference expected from overall body size differences between the sexes. Data on male and female F0 from the same call type that is likely to be produced using the larynx are now required to determine how this large difference between male and female LVF length corresponds to acoustic differences.

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1182

Tables

Table 1. Sexual dimorphism of body size, vocal anatomy and acoustic variables

LVFs = laryngeal vocal folds, VVFs = velar vocal folds, VTL = vocal tract length

oral and nasal VTL measured from the lips and nostrils to the glottis, respectively, with the larynx in its resting state, head angled and pharynx opened. ^ denotes values taken as the midpoint between two measures. Acoustic values from Charlton et al (2011) and Charlton (2015). In all cases sexual dimorphism is calculated by dividing male over female measures.

	Male (n)	Female (n)	Male	Female	Dimorphism
Anatomy					
Body length (mm)	6	6	685	657	1.04
Head length (mm)	6	6	139	127	1.10
LVFs (mm)	10	9	9.8	8	1.22
VVFs (mm)	10	10	32.5	26.2	1.24
oral VTL	1	1	165	142	1.16
nasal VTL	1	1	187	162	1.15
Acoustics					
Bellow mean F0	20	23	28.7	31.3	0.92
Bellow minimum F0	20	23	9.9	12.3	0.80
Bellow ΔF	20	23	354	423	0.84

Figure legends

Fig. 1: Two-stage calling posture of an adult male koala while producing a bellow vocalisation. Initial stretched posture, head, neck, and thorax extended (A); flexed posture towards the end of the call, head angled, neck and thorax less extended (B). Landmarks used for the red longitudinal head line are the mouth opening and the visible ear base, which is close to the atlantooccipital articulation. Landmarks used for the red longitudinal body line are the visible ear base and (less reliable) the assumed position of the cranial edge of the pelvis. In A the head line is in straight continuation of the body line (modified from Charlton et al, 2011)

Fig. 2: Initial calling posture and full larynx retraction demonstrated by a CT-based 3D reconstruction of the skeleton in an adult male specimen. Prior to CT scanning, the larynx had been externally fixed in a maximally retracted position. Left lateral view.

Fig. 3: The nasal cavity of an adult male koala. 3D reconstruction of the skeleton of the upper body; left foreleg, clavicle and shoulder removed; virtual sagittal section of the skull and lower jaw; background: a photo of the same specimen. Tongue, soft palate and hyoid ligament manually reconstructed for full larynx retraction. Left lateral view. Note the presence of a small rostral bone riding on the incisive bone and lending support to the tip of the nose (similar to European wild boar, *Sus scrofa*).

Fig. 4: Fully extended pharynx in an adult male koala. Multi Planar Reconstruction (MPR) based on a CT scan of an adult male, in which the larynx had been externally pushed back and fixed to simulate maximal larynx retraction. The ventral parts of the hyoid apparatus and the larynx are located at the thoracic inlet. Note the capacious naso- and oropharynx and the long soft palate. The IPO (indicated by the red ellipse) is also situated far caudally, opposite the laryngeal entrance.

Fig. 5: Dissection stage at which full retraction of the ventral hyoid parts and the larynx down into the thoracic inlet had been simulated. Maximal extension of the pharynx; the hyoid ligament, and the genioglossolaryngeal muscle are exposed. Scale bar 10 mm

Fig. 6: The excised soft palate of an adult male koala: dorsal view with part of right lateral wall of nasopharynx (A), and ventral view (B). A prominent feature of the dorsal surface is the narrow median trench passing caudally into the IPO. A prominent feature of the ventral surface is the IPO with the large VVFs protruding into the laryngopharynx. The asterisk marks a steel pin inserted into the right blind space lateral to the VVFs. Scale bar 10 mm

Fig. 7: Upper panel: Histological sections of the male VVFs. The cornifying stratified squamous epithelium and the underlying, parallel network of elastic fibres (black) and collagenous fibres (pink) of the upper intermediate layer (A); high density of thicker bundles of collagenous fibres in the lower intermediate layer (B); longitudinally oriented large bundle of elastic fibres, parallel to the free edge of the VVFs in the lower intermediate layer (C); longitudinal muscle fibre bundles of the deep layer near the laterodorsal base of the VVFs (D).

A, B and D: transverse sections, C: sagittal section. A, C and D: Elastica van Gieson staining, D: Masson/Goldner Trichrome staining. In each subfigure the arrow points towards the highlighted feature.

Fig. 7: Lower panel: Histological sections of the female VVFs. The cornifying stratified squamous epithelium and the upper intermediate layer of collagenous and elastic fibres (A); the mostly longitudinally arranged muscle fibre bundles, collagenous and elastic fibres inside the VVF stand out against the mostly transversely oriented thick muscular bundles of the soft palate in the region of the VVFs (B); Detail of the deepest layer of the VVF (C), illustrating the scaffolding of collagenous and elastic fibres interspersed with muscle fibre bundles and mucinous glands; soft palate immediately caudal to the VVFs containing transversely arranged larger bundles of elastic fibres (D).

A, D: transverse sections; B, C: sagittal sections. A, B, C, D: Elastica van Gieson staining. In each subfigure the arrow points towards the highlighted feature.

Fig. 8: CT-based 3D reconstruction of the hyoid apparatus in an adult male koala. The position of the left hyoid ligament is indicated by the yellow bar. The left clavicle, ribs and shoulder girdle have been removed virtually to expose the *in situ* position of the

hyoid apparatus and larynx. The white asterisk marks the cartilaginous connection between the hyoid apparatus and the larynx. The black asterisk marks the cartilaginous longitudinal ventral fusion of thyroid and cricoid cartilages that is typical for marsupials. The resting position of the larynx is approximately at the level of the cervical vertebrae 4-6.

Fig. 9: Superficial muscular triangle covering the ventrolateral neck region, consisting of the two bellies of the digastric muscle rostrally, the sternohyoid muscle ventrally, and the V-shaped omohyoid muscle laterally. A tendinous intersection links these three muscles. Position of lower jaw indicated by white contour. In the koala the omohyoid and sternohyoid muscles have completely lost their connection to the hyoid apparatus. The three muscles are laterally flanking the pharynx, the ventral hyoid parts, the larynx, trachea and oesophagus. Scale bar 10 mm

Fig. 10: Intra-thoracic origin of the sternohyoid and sternothyroid (strap) muscles in a female (A) and a male (B) adult koala. (A) shows the resting position of the larynx whereas in (B) the neck has been extended and the larynx pulled caudally (red arrows) to simulate its retraction down into the thoracic entrance. The origins of the two muscles extend down to the level of the 3rd and 4th costal cartilage. The sternohyoid muscle courses ventrally, contacting the inner thoracic wall, and is covered dorsally by the sternothyroid muscle. *In situ* the intrathoracic portions of both muscles are covered by a sheath of connective tissue. The sternothyroid muscle is the main retractor of the larynx while the sternohyoid muscle, due to its tendinous connection to the digastric and omohyoid muscles, can guide the extensive up-and-down movements of the larynx, the ventral hyoid parts and the pharynx during bellow production. Scale bar 10 mm, respectively

Fig. 11: The hyocephalic muscle in an adult female koala. Left lateral view. The hyocephalic muscle (probably emerging from the fusion of the stylohyoid muscle rostrally, the caudal stylopharyngeal muscle (middle), and the hyopharyngeal muscle caudally) originates from the cerato-, basi- and thyrohyoid and rostrodorsally from the thyroid cartilage, and mainly terminates on the base of the skull, caudally adjacent to the attachment of the hyoid ligament. As a result of this topographical relationships the hypocephalic muscle can assist in protraction of the larynx subsequent to momentary retraction during bellow production. Scale bar 10 mm

Fig. 12: Right half of the larynx of an adult male koala (A) and an adult female koala (B). Medial view. Dissection photo, showing the mucous membrane relief of the laryngeal cavity, including the laryngeal vocal fold (LVF), and the relative positions of the laryngeal cartilages. The thyroid and cricoid cartilages are ventrally fused by a longitudinal cartilaginous keel (A); translucent specimen (B) prepared by using the Spalteholz technique (Spalteholz 1914; Peters 1961; Piechocki 1967). + = rostral horn, o = caudal horn of thyroid cartilage. Scale bars 10 mm, respectively

Fig. 13: Undulating nerves supplying the musculature of the pharynx, which is subjected to considerable length changes during extensive rostrocaudal excursions of the ventral hyoid parts and the larynx. The undulating nerve branches are ensheathed by flexible hose-like connective tissue tubes (three red arrows), which facilitate stretching and rewinding. Asterisks mark two steel pins used for separating the nerves. Left lateral view. Scale bar 10 mm

Fig. 14: Reconstruction of the vocal anatomy of an adult male koala in six layers while producing the very low frequencies during the nasal inhalatory sections of the final stage of bellow vocalisations. The larynx is maximally retracted down into the thoracic inlet; the hyoid ligament is maximally extended and the sternothyroid muscle maximally

contracted. Left lateral view. Parotidoauricularis muscle terminates on sternal manubrium (A); Y-shaped tendinous intersection connects digastric, omohyoid and sternohyoid muscle, the latter with intrathoracic origin (B); the genioglossolaryngeal muscle terminates on pharynx wall, thyrohyoid and thyrohyoid membrane (C); the geniohyoid muscle is markedly elongated and kept in place by passing through an arch formed by the hyoglossus muscle and the rostral branch of the Y-shaped tendinous intersection; the stylohyoid muscle connects the thyrohyoid to the skull base; the sternothyroid muscle has an intrathoracic origin (D); the hyopharyngeus muscle and the caudal constrictors of the pharynx connect the thyrohyoid and the larynx to the pharynx; first rib removed (E); nasal and oral vocal tract, naso- and oropharynx and soft palate maximally extended; VVFs opposite to the laryngeal entrance; complete (longitudinally compressed) trachea from the larynx to the bifurcation (F).